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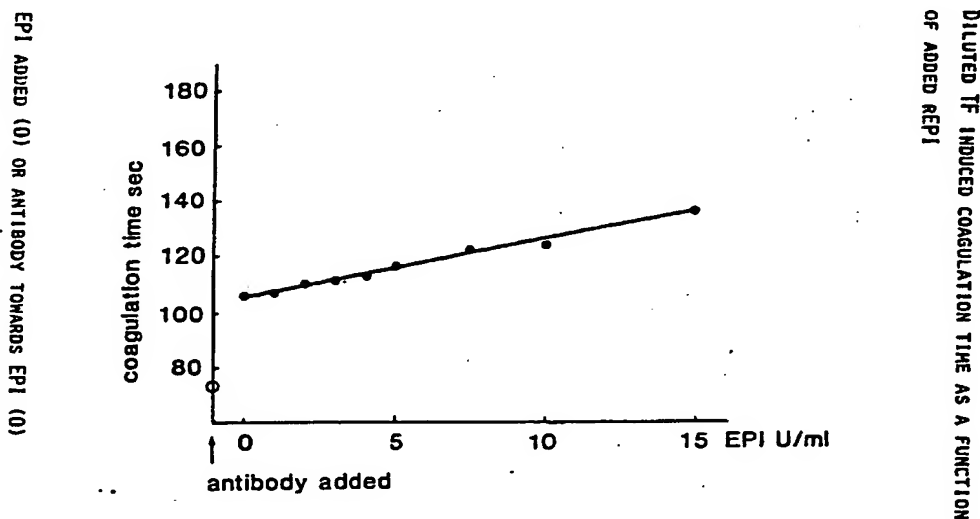
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(54) Title: PHARMACEUTICAL PREPARATION FOR THE TREATMENT OF PROLONGED COAGULATION TIME



(57) Abstract

A pharmaceutical preparation which, as an active component contains an agent that will block EPI activity (EPI inhibitor), is useful in the treatment of patients with prolonged coagulation time.

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#### + DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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PHARMACEUTICAL PREPARATION FOR THE TREATMENT OF PROLONGED  
COAGULATION TIME

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FIELD OF INVENTION

The present invention relates to agents that block EPI activity and the use of these agents to produce preparations that will affect the haemostatic balance.

5 BACKGROUND OF THE INVENTION

Coagulation is a complex process involving many protein factors. The coagulation process can be initiated via the intrinsic pathway through contact with a foreign surface or via the extrinsic pathway through contact with damaged tissue. By 10 extrinsic activation the circulating blood comes in contact with tissue factor (TF). TF is a cofactor for coagulation factor VII (FVII) and the TF-FVII/TF-FVIIa complex activates FX and FIX. Due to the activation of FIX, "intrinsic coagulation" is also influenced by TF activation and an increasing amount of 15 evidence indicate that TF-FVII activation is most important for the initiation of the coagulation reaction (Österud and Rapaport, Proc Natl. Acad Sci USA 74, p 5260, 1977). The coagulation factors of the extrinsic pathway are inhibited by EPI (Extrinsic Pathway Inhibitor) also called LACI (Broze and 20 Miletich, Blood 69, p 150, 1987). EPI is a Kunitz type protease inhibitor which binds and inhibits FXa. The EPI-FXa complex inhibits FVIIa-TF (Rapaport, Blood 73, p 359, 1989). The significance of EPI for the coagulation reaction has not yet been established since only unphysiological high concentrations 25 affects conventional coagulation assays (Broze et al., Biochemistry 29, p 7539, 1990). Therefore, EPI activity is measured in sophisticated assays where EPI is allowed to bind FXa and the EPI-FXa is added to TF-FVII reactants that may activate FIX or FX (Bajaj et al., J Clin Invest 79, p 1974, 30 1987; Sandset et al., Thromb Res 47, p 389, 1987).

In normal haemostasis the coagulation factors are in balance with each other. However, in some circumstances excessive bleeding is observed. Examples are: haemophilia caused by lack of FVIII or FIX, Idiopathic Thrombocytopenia (ITP) caused by

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reduced platelet counts. Also surgery often causes excessive bleeding. Examples of agents that can be used for the prevention of bleeding are: FIX, FVIII, FVIIa, aprotinin and tranexamic acid.

## 5 DISCLOSURE OF THE INVENTION

The present invention shows that agents which block EPI activity will also be able to reduce bleeding tendencies. This is surprising since EPI by itself has not yet been shown to affect the haemostatic balance. However, we have made the following new observations:

1. In coagulation assay plasma EPI is more active than EPI produced by recombinant technique (rEPI).
2. Coagulation induced by very dilute TF is dependent on plasma EPI and thus EPI affects the haemostatic balance.
- 15 3. Antibodies that block EPI activity (anti-EPI) shorten the coagulation time of normal plasma.
4. Anti-EPI shortens the coagulation time of haemophilia plasma.

These observations indicate that blocking of EPI will reduce 20 bleeding tendencies.

Previously it has only been possible to accelerate the coagulation process in normal plasma by adding a coagulation initiator (tissue factor, kaolin, etc.) or an active enzyme (thrombin, FXa, etc). Our experiments provide evidence that it is also 25 possible to accelerate the coagulation process by blocking a plasma protein (EPI).

Intrinsic coagulation is usually measured in APTT assays where coagulation is initiated by Kaolin, phospholipids and  $\text{Ca}^{2+}$ . High concentrations of EPI are needed to affect the APTT assay (Broze, Biochemistry 29,, p 7539, 1990). Extrinsic coagulation is measured in PT assays where coagulation is initiated by tissue factor (brain extract) and  $\text{Ca}^{2+}$ . In the standard assay undiluted tissue factor is used and addition of EPI has only little effect on the coagulation times observed (see example 2). A much more physiological assay is obtained when the tissue factor solution is diluted considerably. Then the coagulation time becomes dependent on extrinsic as well as intrinsic coagulation factors (Brinkhous et al. Proc Natl Acad Sci USA 86, p 1382, 1989). Fig. 1 shows that addition of rEPI increases the coagulation time by approximately two seconds for each EPI unit added. Surprisingly addition of antibody towards EPI (removal of one EPI unit) shorten the coagulation time by 30 seconds. Control experiments were performed to ensure that the effect was really due to inhibition of EPI.

The following results were found:

- 20 A. Addition of excess antibody did not further shorten the coagulation time.
- B. The titer of the antibody in EPI activity inhibition assay corresponds to the titer observed in coagulation time shortening assay.
- 25 C. The antibody was affinity purified by pure rEPI coupled to Sepharose. The resulting IgG preparation showed on a mg basis a 10 fold increase in titer towards coagulation time shortening as well as towards EPI activity.
- D. When antibody was added in an amount just sufficient to shorten the coagulation time then addition of rEPI had a more pronounced effect on the coagulation time increase

compared with addition to plasma without antibody (Fig. 2).

- 5 E. EPI deficient plasma was prepared from normal plasma by immunoabsorption. This plasma had a reduced coagulation time compared with normal plasma. Addition of antibody in the coagulation analysis did not further shorten the coagulation time of the deficient plasma.

Antibodies that block EPI activity have been described in the literature (Novotny et al. Blood 72, p 2020, 1988). However, 10 inhibition of plasma EPI by an antibody has not been described and it has not been observed that such an antibody may have a significant direct effect on coagulation and consequently on the haemostatic balance.

To measure EPI, Novotny et al. use a complicated 3 stage 15 coagulation assay. EPI sample is first incubated with TF, FVIIa, FX and  $\text{Ca}^{2+}$  is added. Then FX is added and after 1 min. incubation FX deficient plasma and rabbit brain cephalin are added. In this assay EPI inhibits the TF activity and it has been shown that anti EPI antibody inhibits the effect of EPI.

20 In a one stage coagulation assay a mixture of  $\text{Ca}^{2+}$  and TF are added directly to plasma and the coagulation time is recorded. In such an assay Broze et al. (Biochemistry 29, p 7543, 1990) found it necessary to add 2.5  $\mu\text{g}$  EPI/ml (50 U/ml) to obtain just 50% reduction in apparent TF activity.

25 Surprisingly we find that adding anti-EPI to plasma (removing 1 U of EPI) significantly affect the coagulation time when coagulation was initiated directly by dilute TF. This coagulation assay mimics the physiological situation since it is the only type of coagulation assay that is dependent on the 30 presence of both FVII, FVIII and FIX all factors that are necessary to obtain normal haemostasis.

Furthermore, plasma EPI has been purified (Novotny et al. J Biol Chem 264, p 18832, 1989). However, it has not been observed that the plasma EPI has a significant effect on coagulation, at least not to such a degree that the inhibition of EPI would affect the haemostatic balance. Thus it has been stressed that documentation for the physiologic importance of EPI remains to be provided (Broze, Biochemistry 29, p 7539, 1990). Our observation, that plasma EPI significantly affects the coagulation time of plasma, provides evidence that EPI has an important role for the haemostatic balance. Furthermore, our experiments provide evidence that blocking of EPI will be able to reduce bleeding tendencies.

#### SUMMARY OF THE INVENTION

In its first aspect the present invention is thus related to a pharmaceutical preparation for the treatment of patients with prolonged coagulation time wherein the preparation as an active component contains an EPI inhibitor.

In a second aspect the invention relates to a method for treating patients with a prolonged coagulation time wherein a preparation, containing an EPI inhibitor, is administered to the patient.

In a third aspect the invention relates to the use of an EPI inhibitor for the production of a pharmaceutical preparation for the treatment of patients with prolonged coagulation time.

#### 25 EXPERIMENTAL PART

Preparation of anti-EPI antibodies: Recombinant human EPI (rEPI) was obtained from transfected BHK cells as described by Pedersen et al. (J Biol Chem, 265, p 6786-6793, 1990). rEPI was purified by heparin affinity chromatography, ion exchange and

reversed phase chromatography (Nordfang et al., Biotech Plasma Prot p 98, 1990). rEPI obtained in this way was pure judged from SDS-PAGE. Rabbits were immunized on day 0, 14, 35 followed by 21 days intervals. Each immunization was with 0.1 mg of rEPI in adjuvans. The first immunization was with Freund's complete adjuvants while the next immunizations were with Freund's incomplete adjuvants. The antisera obtained were tested for inhibition of EPI activity in EPI activity assay and the inhibition was quantitated like FVIII inhibiting antibodies in Bethesda assay: equal volumes of diluted antiserum and EPI (1 U/ml) were incubated for 2 hours at 37°C. EPI activity was measured, and the dilution of antiserum that inhibits the activity by 50% gives the titer. The rabbit antisera had inhibiting titers between 1000 and 4000 "Bethesda-like" units/ml towards both rEPI and human plasma EPI. Below 50 fold dilution serum from unimmunized rabbits did not influence the activity of the EPI sample (1 U/ml). IgG was purified from the antisera by anion exchange chromatography. The IgG preparation with 8 mg IgG/ml contained 2000 "Bethesda-like" inhibiting units/ml towards human plasma EPI.

Assay for EPI activity: EPI was measured in a chromogenic microplate assay, modified after the method of Sandset et al., (Thromb Res 47, P 389, 1989). Heat treated plasma pool was used as a standard. This standard is set to contain 1 U/ml of EPI activity. Standards and samples were diluted in buffer A (0.05 M Tris/0.1 M NaCl/0.1 M Na-citrate/0.02% NaN<sub>3</sub>/pH 8.0) containing 2 µg/ml polybrene and 0.2% bovine serum albumin. FVIIa/TF/-FX/CaCl<sub>2</sub> combination reagent was prepared in buffer A and contained 1.6 ng/ FVIIa (Novo Nordisk A/S), human tissue factor diluted 60 fold (Hjort, Scand J Clin Lab Invest 9, 1957), 50 ng/ml FX (Sigma) and 18 mM CaCl<sub>2</sub>. The assay was performed in microplate strips at 37°C. 50 µl of samples and standards were pipetted into the strips and 100 µl combination reagent was added to each well. After 10 minutes incubation, 50 µl of FX (3.2 µg/ml) was added to each well and after another 10 minutes 25 µl of chromogenic substrate for FXa (S2222) was added 10



minutes after the addition of substrate. The reaction was stopped by addition of 50  $\mu$ l 1.0 M citric acid pH 3.0. The microplate was read at 405 nm.

Coagulation assay: Coagulation activity was measured using an ACL coagulation apparatus. 20  $\mu$ l of antibody solution or EPI solution was incubated with 200  $\mu$ l plasma for 15 minutes at room temperature. After preheating to 37°C the ACL mixes 75  $\mu$ l of plasma sample with 75  $\mu$ l of diluted TF in 20 mM  $\text{CaCl}_2$ , 50 mM NaCl, 17 mM imidazole, 33  $\mu$ g/ml BSA, pH 7.4.

#### 10 EXAMPLE 1

Coagulation assay was performed as described above using 20,000 fold dilution of tissue factor. Table 1 shows the effect of adding anti-EPI to 10 individual donor samples, 7 haemophilia A samples and 1 haemophilia B sample. It appears that a considerable shortening of coagulation time was observed, especially for the haemophilia samples. These data reflect the tentative greater importance of a powerful extrinsic, FVII dependent pathway in the case of an impaired intrinsic coagulation pathway (haemophilia A and B).

20 Table 1. Effect of anti-EPI on the coagulation time of individual plasma samples.

	donors (n=10)	haem A (n=7)	haem B (n=1)
25 Coagulation time without Ab range	102 91-113	131 104-165	165
30 Coagulation time with anti-EPI range	77 71-87	92 82-113	116
35 Reduction, sec. range	26 11-39	39 22-63	49

**EXAMPLE 2**

Coagulation assay was performed with a normal plasma pool using different dilutions of TF. Table 2 shows that the more TF is diluted, the more significant is the effect of adding anti EPI to plasma. This illustrates that the longer the coagulation time, the more effective will anti-EPI be in shortening the coagulation time.

**Table 2**

Effect of adding anti-EPI or EPI as a function of coagulation time.

15	Dilution of TF	Coagulation time in seconds			
		Normal plasma	Normal plasma with anti-EPI	% of NP	NP with 16 U/ml of rEPI add. % of NP
20	60	16.6	16.1	97	17.6 106
	300	28.1	24.5	87	31.4 112
	1,500	49.0	38.9	79	59.0 120
	7,500	92.0	64.8	70	123 134
	15,000	121	80.5	67	>165 >136
25	21,000	143	91.3	64	>165 >
	30,000	>165	102	<62	>165 >
	45,000	>165	117	<	>165 >

**EXAMPLE 3**

Citrate plasma was drawn from a patient after Cardiopulmonary bypass surgery (CPB). In this procedure the patient is anti-coagulated with large doses of heparin. After operation the heparin is neutralized by injection of protamine sulphate. Measured by a chromogenic FXa inhibition analysis the plasma sample contained no heparin. In Sandset EPI assay the sample contained 2 U/ml of EPI activity. Table 3 shows the dilute TF coagulation time of this plasma before and after addition of anti-EPI. It appears that the coagulation time is prolonged and that anti-EPI can shorten the coagulation time. Thus increased

EPI levels may be one of the reasons why CPB patients bleed during and after the operation.

Table 3. Effect of anti-EPI on the coagulation time of plasma from a CPB patient.

5		Donor pool plasma	CPB patient plasma
	Coagulation time without antibody	100	>165
10	Coagulation time with anti-EPI	70	82

#### EXAMPLE 4

15 Heparin binding protein (HBP) WO89/08666 is a protein from neutrophil granula that shows high homology to elastase. HBP is not an enzyme by itself, but seems to bind enzyme inhibitors. When tested in EPI assay the titer of HBP towards EPI is 10 "Bethesda like" units/mg. HBP also blocks the inhibition of FXa  
20 by EPI with a corresponding titer. When added to normal plasma in a concentration of 150 µg/ml, the dilute TF induced coagulation time was shortened (Table 4).

Table 4. Effect of HBP on the coagulation time of normal plasma:

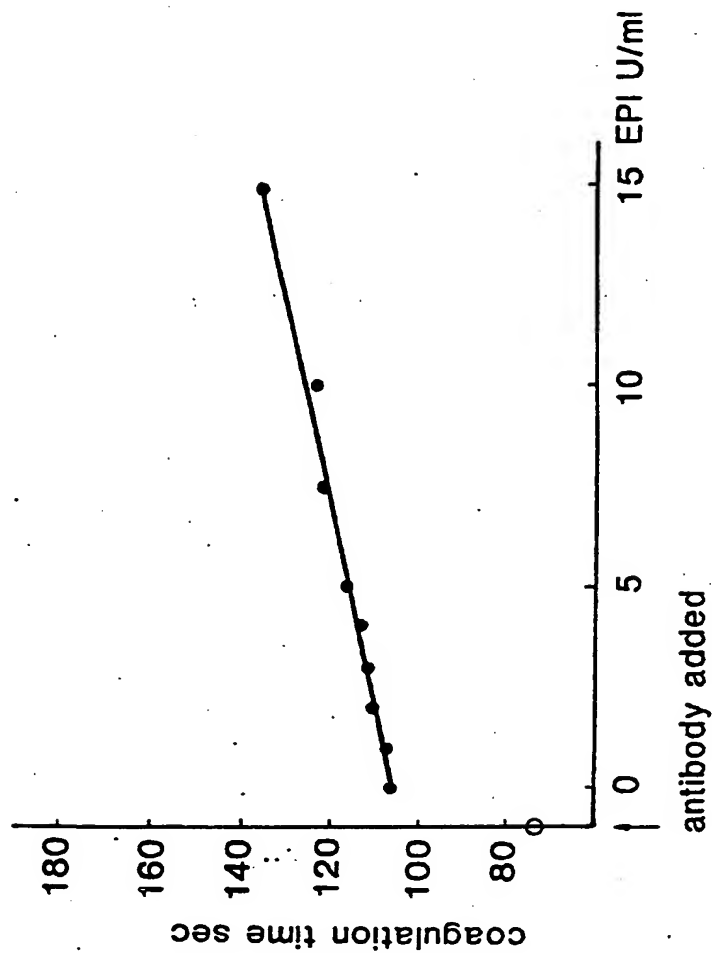
25		Coagulation time, sec.
	Normal plasma	98
30	Normal plasma with anti-EPI	70
	Normal plasma with HBP	81

**CLAIMS**

1. A pharmaceutical preparation for the treatment of patients with prolonged coagulation time c h a r a c t e r i z e d in that the preparation as an active component contains an EPI inhibitor.
2. A pharmaceutical preparation according to claim 1, wherein the coagulation malfunction is due to haemophilia A or B.
3. A pharmaceutical preparation according to claim 1, wherein the coagulation malfunction is due to Idiopathic Thrombocytopenia (ITP), surgery procedures or DIC.
4. A method for treating patients with prolonged coagulation time, which comprises administering to the patient a therapeutically effective dosage of the preparation according to claim 1.
5. A method according to claim 4, wherein the coagulation malfunction is due to haemophilia A or B.
6. A method according to claim 4, wherein the coagulation malfunction is due to Idiopathic Thrombocytopenia (ITP), surgery procedures or DIC.
7. Use of an EPI inhibitor for the production of a pharmaceutical preparation for the treatment of patients with prolonged coagulation time.

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DILUTED TF INDUCED COAGULATION TIME AS A FUNCTION  
OF ADDED REPI

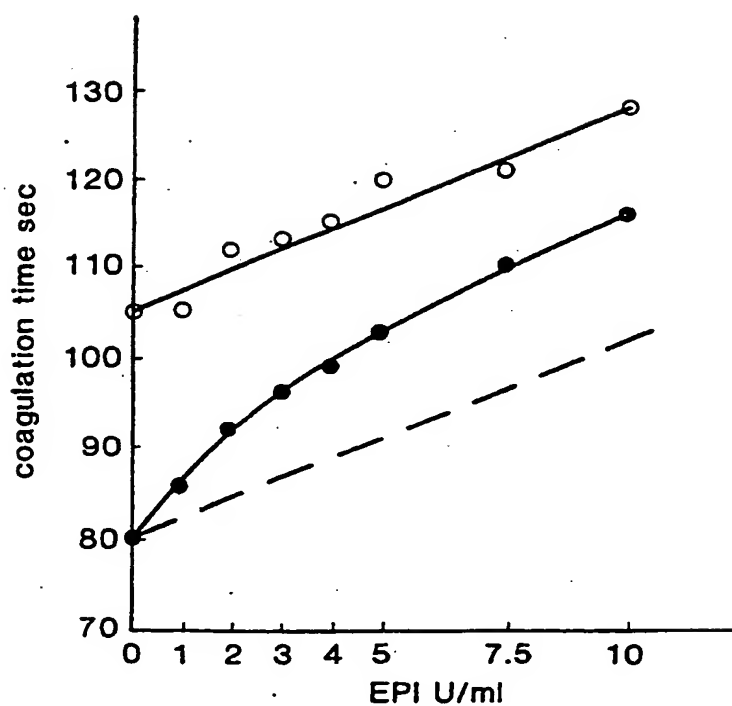


EPI ADDED (O) OR ANTIBODY TOWARDS EPI (O)

FIG. 1

2/2

DILUTED TF INDUCED COAGULATION TIME OF PLASMA AND  
PLASMA WITH ANTI-EPI



REPI WAS ADDED IN DIFFERENT CONCENTRATIONS TO PLASMA.  
THE SAMPLES WERE INCUBATED WITH BUFFER (O) OR WITH  
ANTIBODY TOWARDS EPI (●)

FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00317

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: A 61 K 39/395, 37/64		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC5	A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched <sup>8</sup>		
SE,DK,FI,NO classes as above		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>11</sup>	Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
P,T	THROMBOSIS RESEARCH, Vol. 64, 1991 Dean J. Welsch et al: "Effect of lipoprotein-associated coagulation inhibitor(LACI)on thromboplastin-induced coagulation of normal and hemophilic plasmas", see page 213 - page 222 especially page 220-21 1st paragraph	1-7
P,X	THROMBOSIS RESEARCH Suppl XIV, 1991, Anne Karin Lindahl et al: "Extrinsic pathway inhibitor (EPI) and the post-heparin anticoagulant effect in tissue thromboplastin induced coagulation", see pages 39-48	1,4,6,7
P,X	Dialog Information Services, File 155, Medline 66-92 Dialog accession no. 07885158, Lindahl A K: "Extrinsic pathway inhibitor (EPI) released to the blood by heparin is a more powerful coagulation inhibitor than is recombinant EPI", Thromb Res Jun 15 1991, 62 (6) p 607-14	1,3,7
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
13th February 1992	1992 -02- 14	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	National Library of Medicine, File Medline, Accession no 86221927, Massonnet-Castel S et al: "Partial reversal of low molecular weight heparin (PK 10169) anti-Xa activity by protamine sulfate: in-vitro and in vivo study during cardiac surgery with extracorporeal circulation", Haemostasis 1986; 16 (2): 139-46 ---	1,4,6,7
X	Dialog Information Services, File 155, Medline 66-92 Dialog accession no. 06677231, Sandset PM et al: "Heparin induces release of extrinsic coagulation pathway inhibitor (EPI).", Thromb Res Jun 15 1988, 50 (6) p 803-813 ---	1,4,6,7
A	EP, A2, 0300988 (WASHINGTON UNIVERSITY) 25 January 1989, see page 12 ---	1
A	BIOCHEMISTRY, Vol. 29, No. 33, 1990 George J. Broze, Jr. et al: "Regulation of Coagulation by a Multivalent Kunitz-Type Inhibitor", see page 7539 - page 7546 ---	1-7
A	BLOOD, Vol. 73, No. 2, 1989 Samuel I. Rapaport: "Inhibition of Factor VIIa/Tissue Factor-Induced Blood Coagulation: With Particular Emphasis Upon a Factor Xa-Dependent Inhibitory Mechanism", see page 359 - page 365 ----- ---	1-7



**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 91/00317**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/11/91. The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0300988	89-01-25	AU-B- 604138	90-12-06
		AU-D- 1928688	89-02-02
		JP-A- 1047799	89-02-22
		US-A- 4966852	90-10-30